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Differentially expressed genes associated with post-harvest processing in *Lolium temulentum* L.

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Abstract

Forage and turf grasses are utilized as feed in livestock production, lawns, erosion prevention and recreational surfaces. During grass seed production, grasses in the field are cut while still green and physiologically active. The cut grass is allowed to cure in the fields for 1–2 weeks prior to harvesting the seed. After seed harvest, a large portion of the generated biomass is left as unused or as low value agricultural residue, such as straw. Such biomass is not suitable as animal feedstock and its biochemical composition limits its utility in biofuel conversion efforts. One potential approach to improve the utility of grass residues as feed or biofuels feedstock would be to modify the biochemical or molecular processes within the plant during the period after swathing and prior to seed harvesting. Unfortunately, little knowledge exists regarding post-harvest gene expression in grasses. To this end, we utilized the model grass *Lolium temulentum*, a diploid self-fertile species with a short life cycle (2–3 months), to characterize post-harvest gene expression. A PCR based subtractive suppression hybridization library of a simulated grass straw harvest was prepared and after sequencing a total of 598 unique sequences were identified. Many of these sequences corresponded to orthologs of previously identified genes. The expression patterns of seven genes were evaluated by Northern blot analysis in post-harvest leaf and stem tissues. These preliminary studies support the concept of utilizing of *L. temulentum* as a model forage grass for molecular genetic analyses of post-harvest stress. Published by Elsevier Ireland Ltd.

Keywords: Post-harvest; Straw; Grass; Biofuels; Forage; Subtractive suppression hybridization

1. Introduction

Forage grasses are a major component of the feed used in livestock production worldwide. Turf varieties of these same grasses are utilized for lawns and recreational surfaces and provide major components of buffers that prevent soil erosion. It is estimated that forage grasses are cultivated on over twice the land area used for all other crops worldwide [1]. Forage grass biomass contains nearly as much gross energy by weight as does cereal grain, however, only a fraction (approximately 30–80%) of this energy can be extracted by ruminant animals due to incomplete digestion [2–4]. A large portion of the biomass generated by grass and cereal seed production is left unused or as low value agricultural residues. Such residues include straw from seed production and cuttings from residential lawns and turfs. This biomass is generally not suitable as a high value animal feedstock due to contamination concerns (residential clippings) or to low palatability and nutritional value (straw).

Most current biological (fermentation) approaches to convert grass straws into liquid fuel involve dilute acid pretreatment at elevated temperatures [5,6]. Additionally, treatments with cellulases during the fermentation process can improve the liberation of free sugars and the efficiency of the conversion of plant biomass into biofuels. To be economically feasible, a high degree of efficiency in the pretreatment and saccharification steps are required to release sufficient free sugars for the fermentation process. However, the same properties that make unpalatable biomass sources unsuitable for animal feedstock, such as high lignin content and mechanical resistance to mastication, also restricts the efficiency of saccharification.

One approach for improving the utility of forage grasses would be to alter cell wall composition through genetic modification. Such modification would improve both the nutritional value for animal consumption and the efficiency of the conversion into bio-ethanol. Despite the inherent difficulty,

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decreasing undesirable cell wall components such as lignin by constitutive reduction of lignin synthesis has already resulted in improved forage digestibility [7–9]. However, there are often undesirable morphological changes associated with this approach [3,4,8,10]. Thus, even though it may be desirable to dramatically alter the characteristics of the cell wall (i.e. greatly reduce lignin content) to enhance digestibility some of these modifications would be undesirable if they occurred throughout the entire life cycle of the plant [4,10,11]. Such extensive structural changes could potentially impact stand quality and the upright growth habit of the grass and reduce forage and seed yield. Since many grasses are harvested while the plant is green and still physiologically active, an opportunity exists to alter the molecular composition of the stalks and leaves after cutting by manipulating post-harvest gene expression.

Little knowledge exists regarding changes in gene expression that occur after grasses are cut and harvested. This situation is compounded by the relative difficulty in working with gene expression in forage grasses. The genetic intractability observed in many grasses is due in part to the fact that most are polyploid self-incompatible obligate out-crossers. Despite the observed phenotypic homogeneity displayed in grass populations, they possess a high degree of genetic heterogeneity within populations [12–15]. To further complicate matters, many forage grasses require extensive vernalization to induce flowering. As a consequence, genetic analyses of post-harvest gene expression in many common forage grasses are laborious.

Lolium temulentum L. (Darnel ryegrass) lacks many of the genetic attributes that contribute to the intractability of other grasses to genetic and molecular genetic analyses. It possesses a highly inbred diploid genome and is capable of self-fertilization. Additionally, as a facultative long day plant, it requires no vernalization to complete its life cycle [16,17]. The use of *L. temulentum* as a model grass system has gained momentum and consequently, new tools to enable these analyses are becoming available [18]. Transformation systems are in development, and progress has been made in establishment of embryogenic tissue culture and subsequent regeneration [19]. We utilized this model grass to analyze post-harvest gene expression and conducted suppression subtractive hybridization (SSH) to identify differential expression of genes between pre- and post-harvest grass. These analyses resulted in the identification of genes that are up-regulated in response to post-harvest conditions.

2. Materials and methods

2.1. Plant materials

L. temulentum L. cv. Ceres seeds were planted in approximately one gallon pots in SB40 Sunshine Growing Mix (Sun Gro Horticulture, Canada). Plants were watered daily and fertilized weekly using Technigro 20-18-20 all-purpose fertilizer (Sun Gro Horticulture, Canada). Plants were grown to maturity in a growth chamber that provided 8 h photo-period (short days) at 21 °C day and 18 °C night. Initiation of flowering was performed by adjusting the growth chamber photo-period to 16 h days (long days) and 8 h nights. Mature flowering plants were grown for an additional 3–4 weeks under long days. As the seed heads entered dough stages, watering was reduced to every other day. Seed heads were allowed to ripen until most seed heads were at or beyond the hard dough stage of maturity prior to harvesting.

2.2. Harvest treatments

Harvest treatments were performed to simulate conditions that are commonly observed in grass seed production. Watering was withheld from the mature seed bearing plants for 24 h prior to the harvest date. Grass stalks were cut near ground level and stacked 15–20 cm high in a plastic tray to minimize water evaporation from the edges of the pile, while permitting evaporation from the top of the pile. Tissue samples were collected at 0, 12, 24 and 48 h after cutting. At these time intervals grass samples were taken from the cross-section (from top to bottom) of the stack, the seed heads were removed and the remaining stems and leaf tissue collected. Each tissue sample was quickly cut into 1–2 in. segments, packed in foil, frozen in liquid nitrogen and stored at −80 °C until used.

2.3. RNA isolation

Leaf and stem tissues were ground to a powder using a mortar and pestle cooled with liquid nitrogen. Total RNA was extracted from the ground tissues using TRIzol reagent (Invitrogen, USA) following manufacturer instructions. The total RNA was used for production of the suppression subtractive hybridization library and subsequent Northern blot analysis. Poly(A+) RNA was isolated from 1 mg of total RNA from pre- (0 h after harvest) and post-harvest (a pool of total RNA isolated at 12, 24 and 48 h post-harvest and used for suppression subtractive hybridization.

2.4. Suppression subtractive hybridization

The subtraction library was prepared using a PCR-Select cDNA subtraction kit (Clonetech, CA) according to the manufacturers' instructions. Tester and driver cDNAs were prepared from 2 μg of mRNA extracted from post- and pre-harvest tissues, respectively. The resulting PCR products were subsequently ligated into vector using the pGEM T Easy "TA" cloning kit (Promega, USA) according to the manufactures recommendations.

2.5. Library preparation and sequencing

The ligation products of the SSH library were used to transform competent *Escherichia coli* cells (TOP 10; Invitrogen, USA). Approximately 768 well-separated white colonies were selected for sequencing. The library was maintained in eight 96 well plates. One replica set was shipped to Rexagen Inc. (WA, USA) for sequencing performed with an automatic

Table 1
cDNA clones isolated from a subtractive hybridization library of post-harvest stressed plants

Sequence ID	Clones contained	Length	Annotation	Top Blastx alignment			Top rice reference alignment		
				Accession	Score	Value	Accession	Score	Value
Detoxification									
Cluster 1[a]	4	414	Catalase	AAC17729	165	4.00E-040	XP_470174	133	3.00E-032
SPH C59	1	599	Glutathione S-transferase	AAM89393	185	8.00E-046	NP_922457	172	1.00E-043
SPH_C531	1	499	Manganese Superoxide Dismutase	AAX68501	253	2.00E-066	XP_550626	73	1.00E-013
Cluster 2[a]	**3**	**221**	**Metallothioneine**	**CAD54078**	**62**	**7.00E-009**	**XP_478323**	**28**	**1.6**
SPH C54	2	608	Oxidoreductase Iron/Ascorbate Family	XP_467483	250	3.00E-065	XP_467483	249	1.00E-066
SPH C166	1	177	Oxidoreductase Iron/Ascorbate Family ds3	XP_476744	63	4.00E-009	XP_476744	63	5.00E-011
SPH_C557	1	393	Thioredoxin-Like PKCq-lnteracting Protein	NP_922186	191	1.00E-047	NP_922186	191	1.00E-049
Energy transfer									
Cluster 3[a]	2	491	Ferredoxin	AAU43972	281	6.00E-075	NP_916772	214	2.00E-056
SPH C196	1	712	NADH Dehydrogenase	X90650	279	1.00E-075	X90650	532	e-150
SPH C324	1	735	Plasma Membrane H+-ATPase	AAV71150	427	e-118	XP_474175	412	e-116
SPH C148	1	310	Vacuolar ATP Synthase Subunit H	NP_910644	196	2.00E-049	NP_910644	196	2.00E-051
SPH_C72	1	548	Vacuolar Proton-ATPase	BAB18682	217	1.00E-055	XP_476022	29	1.9
General metabolism									
SPH C7	1	736	3-ketoacyl-CoA Thiolase	XP_468412	336	4.00E-091	XP_468412	330	5.00E-091
SPH C416	1	411	4-coumarate-CoA Ligase Catty-acyl-CoA Synthase	NP_192425	171	1.00E-041	XP_482683	131	1.00E-031
SPH C95	1	487	4-methyl-5(B-hydroxyethyl)-thiazol Ponophosphate Synthase	BAD54224	276	2.00E-073	NP_913360	186	6.00E-048
SPH C76	**4**	**524**	**6-phospho-1-fructokinase**	**XP_467672**	**331**	**S.00E-090**	**XP_467672**	**331**	**2.00E-091**
SPH_C358	1	685	6-phosphogluconolactonase	XP_483640	163	4.00E-039	XP_483640	163	7.00E-041
Cluster 4[a]	5	730	Aconitate Hydratase	XP_474477	443	e-123	XP_474477	443	e-125
SPH C221	2	622	ACT Domain Protein	ABA95833	374	e-102	NP_909842	60	1.00E-009
SPH C565	1	361	Acyltransferase	NP_919461	192	4.00E-048	NP_919461	192	5.00E-050
SPH C368	2	615	Alanine-glyoxylate Aminotransferase	AAU44251	389	e-107	XP_477982	92	2.00E-019
SPH C140	2	337	Alcohol Dehydrogenase	CAA34547	83	4.00E-015	NP_912565	52	8.00E-008
SPH C174	1	739	Aldehyde Dehydrogenase	AAG43027	259	8.00E-068	XP_472778	97	1.00E-020
SPH C63	1	585	Alpha-glucosidase	AAF76254	335	7.00E-091	NP_909121	191	3.00E-049
SPH C567	1	356	Arabinoxylan Arabinofuranohydrolase Isoenzyme	AAK21880	242	3.00E-063	XP_471515	206	2.00E-054
SPH C382	2	569	Aspartate Aminotransferase	XP_463436	366	e-100	XP_463436	366	e-102
SPH C206	1	695	ATP sulfurylase	AAB94542	418	e-116	XP_469693	418	e-117
SPH C173	1	740	Beta 1-3 Glucan Synthase	XP_550490	464	e-129	XP_550490	464	e-131
SPH C398	1	487	Beta-D-glucan Exohydrolase Isoenzyme	AAD23382	247	1.00E-064	XP_469757	247	3.00E-066
SPH C40	2	681	CBS Domain Containing Protein	XP_506633	240	3.00E-062	XP_506633	241	2.00E-064
SPH C250	1	501	Ceramidase	NP_917762	258	6.00E-068	NP_917762	260	3.00E-070
SPH C539	1	462	Choline-phosphate Cytidylyltransferase	XP_464309	111	1.00E-023	XP_464309	111	2.00E-025
SPH C326	1	735	Cinnamoyl-CoA Reductase	XP_474491	99	2.00E-019	XP_474491	99	3.00E-021
SPH C371	1	614	Cinnamyl Alcohol Dehydrogenase	BAD73514	366	e-100	NP_918057	366	e-102
SPH C35	1	700	Citrate Synthase	AAG28777	398	e-109	XP_464443	396	e-111
SPH C26	1	714	Copper Oxidase Laccase	CAJ30499	381	e-104	NP_918753	334	3.00E-092
SPH C545	1	432	Cytochrome B5	NP_914346	134	1.00E-030	NP_914346	134	3.00E-032
SPH C513	1	546	Cytochrome B6	BAD72231	345	5.00E-094	NP_914558	340	2.00E-094
Cluster 5[a]	2	696	Cytochrome P450	XP_479692	261	1.00E-068	XP_479692	261	2.00E-070
SPH C253	1	487	Dehydroquinate Synthase	BAD46567	258	5.00E-068	XP_474490	28	3.4
SPH C97	1	483	Deoxycytidine Deaminase	XP_470319	275	3.00E-073	XP_470319	275	7.00E-075
SPH C468	1	747	Dihydropyrimidine Dehydrogenase	AAM94290	331	1.00E-089	NP_919490	324	4.00E-089
SPH C593	1	213	Ethylene-forming Dioxygenase-like enzyme	XP_476310	102	3.00E-021	XP_476310	102	4.00E-023
SPH C544	1	440	Ferric Reductase-like	XP_478984	129	4.00E-029	XP_478984	129	9.00E-031
SPH C127	1	403	Flavonol 4′-sulfotransferase	XP_450330	136	3.00E-031	XP_450330	136	4.00E-033
SPH C238	1	520	Flavonol/Cinnamoyl-CoA Reductase	BAD73619	223	2.00E-057	NP_915311	223	5.00E-059
SPH C306	1	201	Fructose 1-6-biphosphate Aldolase	CAD12665	133	2.00E-030	XP_479829	110	3.00E-025
SPH C119	1	426	Fumarate Hydratase Interacting Protein FIP1	NP_565538	136	3.00E-031	NP_912553	134	2.00E-032
SPH_C282	2	348	Galactose Kinase	XP_470165	173	2.00E-042	XP_470165	173	2.00E-044
Cluster 6[a]	2	326	Galactosyltransferase	XP_475253	117	1.00E-025	XP_475253	117	1.00E-027
SPH C194	1	716	Glutamate Dehydrogenase 2	BAE48298	283	4.00E-075	XP_473381	276	8.00E-075
SPH C106	5	478	Glutamine-dependent Asparagine Synthetase	AAU89392	273	2.00E-072	XP_476370	32	0.14
SPH C410	2	429	Glyoxysomal Citrate Synthase	BAD27711	278	3.00E-074	XP_464443	39	9.00E-004
SPH C21	1	716	Hexokinase 6	AAX68422	359	6.00E-098	XP_476073	349	8.00E-097
SPH C134	1	368	Homogentisate 12-dioxygenase	BAD67951	106	3.00E-022	NP_921882	34	0.02

Table 1 (*Continued*)

Sequence ID	Clones contained	Length	Annotation	Top Blastx alignment			Top rice reference alignment		
				Accession	Score	Value	Accession	Score	Value
SPH C322	1	739	Hydroxyanthranilate Hydroxycinnamoyltransferase AsHHT-like	XP_473296	114	3.00E-024	XP_473296	114	6.00E-026
SPH C158	1	220	Isom erase	NP_564250	147	9.00E-035	XP_471059	46	6.00E-006
SPH C501	1	674	Leucoanthocyanidin Dioxygenase	AAX95314	184	2.00E-045	XP_475566	169	1.00E-042
SPH C542	**1**	**453**	**Lipoxygenase**	**P29114**	**191**	**6.00E-04S**	**XP_469409**	**168**	**1.00E-042**
SPH C387	1	554	Long Chain Acyl-CoA Synthetase	AAV44023	353	1.00E-096	NP_916942	119	8.00E-028
SPH C4	1	739	Lysine Ketoglutarate Reductasesaccharopine Dehydenase	AAG21985	343	3.00E-093	XP_467029	30	1.1
SPH C214	1	646	Myo-inositol Phosphate Synthase	AAN52772	383	e-105	NP_921086	313	5.00E-086
SPH C37	1	697	Myotubularin Related Protein	AAF76357	186	8.00E-046	XP_473469	35	0.05
SPH C223	1	624	N-acetylglucosamine-phosphate Mutase	XP_476984	313	2.00E-084	XP_476984	313	4.00E-086
SPH C242	1	514	N-methyltransferase	ABA96840	321	6.00E-087	XP_467559	56	1.00E-008
SPH C168	1	744	NAD Dependent Epimerasedehydratase	ABA94522	471	e-131	NP_921492	459	e-129
SPH_C200	1	703	NADP-specific Isocitrate Dehydrogenase	NP_917313	363	4.00E-099	NP_917313	363	e-101
Cluster 7[a]	7	664	NADP+-dependent Malic Enzyme	NP_914533	382	e-105	NP_914533	382	e-106
SPH C142	1	334	Phosphatidylinositol Transfer	XP_467526	137	1.00E-031	XP_467526	137	1.00E-033
SPH C108	1	468	Phosphatidylserine Decarboxylase	XP_471507	115	5.00E-025	XP_471507	115	1.00E-026
SPH C107	1	470	Phospholipase D	XP_450186	298	4.00E-080	XP_450186	298	1.00E-081
SPH C547	1	418	Phosphomannomutase	XP_474395	255	3.00E-067	XP_474395	255	5.00E-069
SPH C471	1	746	Prephenate Dehydratase	XP_479626	72	1.00E-011	XP_479626	72	3.00E-013
SPH C330	1	730	Reductase	BAD82407	291	2.00E-077	NP_913460	233	8.00E-062
SPH C145	3	331	Saccharopin Dehydrogenase-like	CAD48130	92	6.00E-018	XP_468135	89	8.00E-019
SPH C163	2	197	Spermidine Synthase	AAW57523	129	3.00E-029	XP_507360	109	4.00E-025
SPH_C172	1	740	Sucrose Synthase	AAK52129	251	1.00E-065	NP_909830	251	2.00E-067
Cluster 8[a]	2	737	Sucrose Synthase 3	AAM89473	433	e-120	NP_914696	340	4.00E-094
SPH C394	1	505	Sucrosephosphate Synthase	AAQ56529	160	2.00E-038	XP_481429	160	5.00E-040
SPH C469	1	746	Transaldolase	BAD88191	402	e-111	XP_463680	402	e-112
SPH C41	2	675	Tyrosine Aminotransferase	ABA95087	260	3.00E-068	XP_465161	131	3.00E-031
SPH_C131	1	370	UDP-glucose:sterol glucosyltransferase	CAB06081	244	6.00E-064	XP_471170	243	3.00E-065
Maintenance genes									
SPH C293	2	285	Actin	AAU44177	83	3.00E-015	NP_914272	83	3.00E-017
SPH C490	1	727	Actin Bundling Protein	AAD54660	345	9.00E-094	XP_473786	270	6.00E-073
SPH C269	2	421	Alpha-Tubulin 3	CAA62916	169	3.00E-041	XP_507378	162	5.00E-041
SPH C331	1	730	ATP-dependent RNA helicase	BAD88051	203	4.00E-051	NP_918275	202	2.00E-052
SPH_C555	1	396	Cullin-like Protein	BAD61452	205	4.00E-052	NP_918711	205	6.00E-054
Cluster 9[a]	2	547	Cyclin CLB1 Protein	BAD46564	296	2.00E-079	NP_915992	266	4.00E-072
SPH C584	1	260	Exonuclease	NP_921413	125	5.00E-028	NP_921413	125	6.00E-030
SPH C80	1	529	Exonuclease Phosphatase	BAD52532	179	5.00E-044	NP_921451	30	0.8
SPH C564	1	361	Histone H2A	AAS20970	117	1.00E-025	XP_482492	109	4.00E-025
SPH_C117	1	442	Hydroxyproline-rich GlycoProtein	XP_476783	138	7.00E-032	XP_476783	138	1.00E-033
Cluster 10[a]	3	354	Leucine Rich Repeat Protein LOC	NP_915064	224	1.00E-057	NP_915064	224	1.00E-059
SPH C2	1	742	Myosin-like Protein	NP_919960	276	5.00E-073	NP_919960	276	9.00E-075
SPH C274	1	393	Nuclease I	XP_550001	224	1.00E-057	XP_550001	224	2.00E-059
SPH C185	1	730	Nucleotide 5′ Nucleotidase Family	AAL32602	323	4.00E-087	NP_912742	262	1.00E-070
SPH C444	1	271	Plant lipid transfer Protein	AAB70539	87	3.00E-016	XP_475420	59	9.00E-010
SPH C503	1	621	Profilin	XP_550652	231	1.00E-059	XP_550652	231	2.00E-061
SPH C470	1	746	Prosaposin	ABA91163	246	4.00E-064	XP_550252	143	1.00E-034
SPH C222	1	628	RNase H	AAX95607	133	4.00E-030	XP_462939	133	7.00E-032
SPH C44	1	664	S6 Ribosomal Protein Kinase	CAA56313	428	e-119	XP_479548	390	e-109
SPH C475	1	740	Seed Maturation Protein PM23	AAR87215	397	e-109	XP_463125	397	e-111
SPH C197	1	710	SYD chromatin remodeling ATPase	AAV70959	187	4.00E-046	NP_920532	34	0.07
SPH C313	2	712	tRNA-Asn	CAJ00817	90	9.00E-017	No hits found	na	na
SPH C116	5	436	tRNA-Gly	ZP00422983	37	0.18	XP_493800	27	6.1
SPH C422	1	385	tRNA-Leu	AAO74089	56	4.00E-007	NP_909475	27	4.4
SPH_C272	1	404	tRNA-Ser	XP_465410	57	2.00E-007	XP_465410	57	3.00E-009
Photosynthesis									
SPH C74	1	543	Chlorophyll ab Binding Protein	AAF26741	294	1.00E-078	NP_916688	291	2.00E-079
SPH_C328	1	734	Rubisco	AAA32046	105	2.00E-021	NP_039391	99	2.00E-021
Plastid/mitochondrial genome									
SPH C446	1	264	ATP synthase 1 subunit	AAS46117	163	2.00E-039	NP_920963	163	2.00E-041
SPH C36	1	698	ATP Synthase Alpha Subunit	AAS46118	48	4.00E-004	XP_475046	48	6.00E-006
Cluster 11[a]	3	468	ATP Synthase Beta Subunit	AAT44700	265	4.00E-070	NP_039390	264	2.00E-071

Table 1 (*Continued*)

Sequence ID	Clones contained	Length	Annotation	Top Blastx alignment			Top rice reference alignment		
				Accession	Score	Value	Accession	Score	Value
SPH C189	1	727	Chloroplast Protease	BAD45447	349	5.00E-095	NP_918496	117	5.00E-027
SPH C135	1	367	Chloroplast Ptr ToxA-binding Protein	AAR24582	113	2.00E-024	XP_478693	103	2.00E-023
SPH C53	3	614	ChloroplastictRNA-Met	ZP_01055282	34	4.1	NP_921318	27	7
SPH C435	1	329	Cytochrome b6	AAT44721	222	4.00E-057	NP_915746	222	5.00E-059
SPH C350	2	697	Cytochromef	AAS46130	338	8.00E-092	NP_920976	335	1.00E-092
SPH C100	1	480	Mitochondrial Aldehyde Dehydrogenase	BAB62757	270	1.00E-071	XP_467608	233	5.00E-062
Cluster 12[a]	2	715	Mitochondrial Carrier Protein	XP_475118	245	9.00E-064	XP_475118	245	2.00E-065
SPH C354	1	690	NADH Dehydrogenase Subunit 2	CAA60363	206	4.00E-052	XP_481013	206	1.00E-053
SPH C479	1	737	NADH Dehydrogenase Subunit 4	CAA31558	330	2.00E-089	NP_039444	299	1.00E-081
SPH C64	1	583	NADH Dehydrogenase Subunit 4L	CAA09815	101	2.00E-020	NP_910143	100	1.00E-021
SPH C188	2	721	NADH Dehydrogenase Subunit 5	AAS46154	134	2.00E-030	NP_039441	134	4.00E-032
SPH C191	1	725	Photosystem I Assembly Protein Ycf3	CAA60287	96	1.00E-018	NP_039384	92	2.00E-019
SPH C301	1	257	PsaB Photosystem I	AAT00783	168	7.00E-041	NP_920966	167	2.00E-042
SPH C336	1	726	psbB Chlorophyll-binding Protein	CAA30519	213	5.00E-054	NP_920978	208	3.00E-054
SPH C478	1	737	Ribosomal Protein L2	AAR91063	336	4.00E-091	NP_039427	236	1.00E-062
SPH C141	1	339	Ribosomal Protein S11	NP_114290	129	4.00E-029	NP_039418	126	3.00E-030
Cluster 13[a]	4	683	Ribosomal Protein S12	CAA30522	131	2.00E-029	NP_039359	119	1.00E-027
SPH C556	1	394	Ribosomal Protein S14	NP_114257	135	5.00E-031	NP_922435	132	8.00E-032
SPH C579	2	291	Ribosomal Protein S15	ABA98782	120	2.00E-026	NP_920980	119	3.00E-028
SPH C210	1	673	Ribosomal Protein S16	CAD59435	43	0.01	NP_920950	31	0.55
SPH C198	2	699	Ribosomal Protein S18	NP_114280	157	3.00E-037	NP_039408	155	2.00E-038
SPH C339	1	722	Ribosomal Protein S4	NP_114261	255	9.00E-067	NP_922432	243	8.00E-065
SPH C518	1	535	Ribosomal Protein S50	Q95H50	135	5.00E-031	NP_039423	133	5.00E-032
SPH C247	1	510	Ribosomal Protein S7	AAR91190	295	5.00E-079	XP_481012	293	3.00E-080
SPH C462	1	756	Ribosomal Protein S8	NP_114293	209	8.00E-053	XP_481019	205	3.00E-053
Cluster 14[a]	5	720	RNA polymerase subunit beta	BAA78042	410	e-113	NP_039375	358	2.00E-099
Cluster 15[a]	10	547	Unidentified Plastid Genome Match	CAA60360	158	1.00E-037	NP_039455	144	3.00E-035
Protein degradation, folding and transport									
SPH C568	1	353	26S Proteasome Non-ATPase Regulatory Subunit	AAV31238	105	7.00E-022	NP_912909	105	8.00E-024
SPH C233	1	543	26S Proteasome Subunit	NP_918654	282	4.00E-075	NP_918654	282	7.00E-077
SPH_C62	1	586	Aminopeptidase P	ABA94110	300	2.00E-080	XP_477069	99	2.00E-021
Cluster 16[a]	**2**	**705**	**ATP-dependent Clp Protease ATP-binding Subunit**	**AAN78327**	**146**	**7.00E-034**	**XP_466044**	**146**	**1.00E-035**
SPH C367	1	625	ATP-dependent Zn protease	CAA62084	364	1.00E-099	NP_915446	193	6.00E-050
SPH C379	1	575	Clp Amino Terminal Domain	ABA96309	256	2.00E-067	XP_472335	204	2.00E-053
Cluster 17[a]	2	728	ClpA ATPaseswith Chaperone Activity	ABA96309	419	e-116	XP_472335	409	e-115
Cluster 18[a]	2	741	Cysteine Protease Cathepsin B	CAC83720	330	3.00E-089	NP_914345	50	2.00E-006
Cluster 19[a]	**3**	**537**	**Cysteine Proteiase C1 Papain family**	**BAA14402**	**308**	**8.00E-083**	**XP_474131**	**302**	**7.00E-083**
Cluster 20[a]	2	452	Cysteine Proteinase	CAB71032	291	4.00E-078	XP_467463	120	3.00E-028
SPH C515	**1**	**543**	**Cysteine Proteinase Inhibitor**	**BAB1S76S**	**171**	**2.00E-041**	**NP_915842**	**139**	**8.00E-034**
Cluster 21[a]	2	735	FtsH Protease	NP_918496	356	5.00E-097	NP_918496	356	9.00E-099
Cluster 22[a]	**2**	**630**	**LON1 Protease**	**AAK62365**	**389**	**e-107**	**NP_910416**	**128**	**3.00E-030**
SPH C396	**1**	**497**	**Metal loprotease**	**BAD293S2**	**288**	**7.00E-077**	**NP_917058**	**28**	**2.1**
SPH_C121	1	420	Peptidase S10 Serine Carboxypeptidase	XP_507511	278	3.00E-074	XP_507511	278	6.00E-076
Cluster 23[a]	**14**	**636**	**Peptide Chain Release Factor**	**XP_469434**	**393**	**e-108**	**XP_469434**	**393**	**e-110**
Cluster 24[a]	2	716	Polyubiquitin	CAA66667	454	e-126	XP_506723	454	e-128
Cluster 25[a]	2	715	Protein phosphatase type 2C	AAK20060	431	e-120	NP_922589	431	e-121
SPH C476	1	739	Serine Carboxypeptidase III	P11515	356	5.00E-097	XP_463859	332	2.00E-091
SPH C137	1	352	Serine Peptidase	AAK84459	216	2.00E-055	NP_922290	216	2.00E-057
SPH C427	2	368	Serine Protease Subtilase-like	NP_915782	181	8.00E-045	NP_915782	181	1.00E-046
SPH C85	1	509	Serine Protease Subtilisin-like	BAD53015	251	1.00E-065	NP_915781	251	2.00E-067
SPH C240	1	515	Ubiquinol-Cytochrome-C Reductase-like Protein	CAB87150	234	1.00E-060	XP_466001	229	7.00E-061
SPH C130	1	374	Ubiquitin Conjugating Enzyme	BAD35271	250	1.00E-065	XP_474269	52	1.00E-007
SPH_C280	1	357	Xaa-Pro dipeptidase	XP_464731	214	6.00E-055	XP_464731	214	7.00E-057
Ribosomal									
SPH C286	1	336	ribosomal Protein S14	XP_464199	158	5.00E-038	XP_464199	158	6.00E-040
SPH C467	1	749	Ribosomal Protein S16	XP_481017	125	1.00E-027	XP_481017	125	2.00E-029
SPH C167	1	745	Ribosomal Protein S23	CAA55027	193	6.00E-048	NP_039462	192	2.00E-049
Cluster 26[a]	3	571	Ribosomal RNA 23S	AAO74082	50	4.00E-005	XP_482433	43	1.00E-004

Table 1 (*Continued*)

Sequence ID	Clones contained	Length	Annotation	Top Blastx alignment			Top rice reference alignment		
				Accession	Score	Value	Accession	Score	Value
Signal transduction									
SPH C178	1	737	AarF Predicted Unusual Protein Kinase	AAK63973	393	e-108	XP_468400	212	2.00E-055
SPH C71	1	555	CBL-interacting Protein Kinase	XP_476651	137	2.00E-031	XP_476651	137	3.00E-033
SPH C452	1	242	Protein Kinase-like	XP_468268	109	4.00E-023	XP_468268	109	4.00E-025
SPH_C58	1	602	Protein Phosphatase Type 2C	XP_472380	208	1.00E-052	XP_472380	208	2.00E-054
Cluster 27[a]	3	698	Receptor-like Protein Kinase	BAD81104	265	1.00E-069	NP_912761	265	2.00E-071
Cluster 28[a]	5	742	Ser/Thr Protein Kinase	XP_466474	417	e-115	XP_466474	417	e-117
SPH C46	1	651	Ser/Thr Protein Phosphatase	NP_917035	340	2.00E-092	NP_917035	340	4.00E-094
SPH C295	1	283	Small Ran-related GTP-binding Protein	AAM08320	91	2.00E-017	XP_475914	85	9.00E-018
SPH_C380	1	574	Tyrosine Protein Kinase	XP_469008	343	3.00E-093	XP_469008	343	5.00E-095
Stress related									
SPH C259	1	456	14-3-3 Protein	CAA44259	239	3.00E-062	XP_469508	238	1.00E-063
SPH C522	1	520	Callose Synthase-like Protein	AAP84973	318	4.00E-086	XP_468556	318	8.00E-088
SPH C373	1	607	Chitinase Class III	NP_921480	161	1.00E-038	NP_921480	161	2.00E-040
SPH C566	1	360	Dehydrin	AAV88600	52	7.00E-006	NP_917108	29	0.73
SPH_C528	1	504	Dehydrin OSOSR40C1	CAA64683	149	4.00E-035	NP_912421	149	8.00E-037
Cluster 29[a]	**8**	**555**	**Dehydrin rab25**	**CAA33364**	**72**	**1.00E-011**	**NP_917108**	**31**	**0.52**
SPH C529	1	501	Electron Transporter/Heat Shock Binding	AAU43972	288	5.00E-077	NP_916772	226	4.00E-060
SPH C585	1	260	Hsp70 Binding Protein	NP_910052	149	3.00E-035	NP_910052	149	4.00E-037
SPH C216	**1**	**640**	**Jasmonate Induced Protein**	**CAA58110**	**243**	**5.00E-063**	**XP_471759**	**92**	**3.00E-019**
SPH C415	1	418	LEA2 Protein	BAC80266	65	1.00E-009	XP_475821	55	1.00E-008
Cluster 30[a]	2	743	Osmotic Stress-activated Protein Kinase	XP_473504	327	3.00E-088	XP_473504	327	6.00E-090
SPH C224	**2**	**604**	**Senescence-associated Protein**	**AAV31120**	**176**	**6.00E-043**	**XP_482646**	**162**	**2.00E-040**
SPH_C276	1	377	Universal Stress Protein	AAV65310	158	7.00E-038	XP_479478	157	1.00E-039
Transcription factor									
SPH C401	1	478	Auxin-responsive Factor	CAE04227	181	1.00E-044	XP_466220	150	3.00E-037
SPH C558	1	393	DnaJ Domain Protein	NP_922851	137	2.00E-031	NP_922851	137	2.00E-033
SPH C78	1	531	Global Transcription Factor Group C	AAN41252	239	4.00E-062	XP_471832	228	1.00E-060
SPH C360	1	666	Initiation Factor 4E p26	CAA78262	316	4.00E-085	NP_914338	308	1.00E-084
SPH C534	1	481	NAC Domain Transcription Factor	NP_912423	80	3.00E-014	NP_912423	80	6.00E-016
SPH C297	1	282	NAM (No Apical Meristem) Protein	XP_473174	138	7.00E-032	XP_473174	138	9.00E-034
SPH C409	1	437	PHD Finger Protein-like	XP_464577	163	2.00E-039	XP_464577	163	4.00E-041
SPH C424	1	381	Potato Inhibitor I Family	AAX95304	92	5.00E-018	XP_482622	70	4.00E-013
SPH C426	1	371	PRLI-interacting factor K	BAD87678	194	7.00E-049	NP_918120	194	8.00E-051
SPH C102	1	476	SABRE-like Protein	DAA00365	141	7.00E-033	XP_480538	28	1.9
SPH C89	1	499	Scarecrow-like Protein	NP_915059	310	2.00E-083	NP_915059	310	4.00E-085
SPH C332	1	729	Splicing Factor CC1-like	ABB47695	284	2.00E-075	NP_921672	242	1.00E-064
SPH_C407	1	445	Transcriptional Co-repressor-like	XP_549869	165	4.00E-040	XP_549869	165	9.00E-042
Cluster 31[a]	5	423	Translation Initiation Factor	XP_450544	215	5.00E-055	XP_450544	215	9.00E-057
SPH_C267	**2**	**429**	**Zn ribbon-containing Protein**	**NP_917975**	**150**	**1.00E-035**	**NP_917975**	**150**	**2.00E-037**
Transport and membrane bound									
SPH_C258	1	458	Auxilin-like Protein	ABA95242	191	7.00E-048	NP_918860	149	9.00E-037
Cluster 32[a]	4	654	Ca+2 Trasporting ATPase	CAC40034	396	e-109	XP_493908	371	e-103
SPH C96	1	484	GDP Dissociationl Inhibitor	AAV25637	307	1.00E-082	XP_477386	275	1.00E-074
SPH C361	1	666	Golgi SNAP Receptor Complex Member 1	BAD29263	320	2.00E-086	XP_482607	319	1.00E-087
SPH C571	1	348	Graves Sisease Mitochondrial Solute Carrier Protein	BAD81517	214	6.00E-055	XP_463329	178	6.00E-046
SPH C125	1	403	GTP-binding Protein (rab2)	AAW52512	175	4.00E-043	XP_466431	174	2.00E-044
SPH C340	1	721	Hexose Transporter	XP_464773	397	e-109	XP_464773	397	e-111
SPH C16	1	721	Histidine Amino Acid Transporter	CAD89802	223	4.00E-057	XP_479903	223	7.00E-059
Cluster 33[a]	3	515	Membrane Protein	CAA33959	80	4.00E-014	NP_039397	80	9.00E-016
SPH C526	1	518	Membrane Protein MtN21-like	XP_483787	109	5.00E-023	XP_483787	107	4.00E-024
SPH C8	1	735	Mitochondrial Carrier Protein	NP_912344	355	1.00E-096	NP_912344	355	2.00E-098
SPH C570	1	350	Monosaccharide Transporter 4	AAQ24871	129	3.00E-029	NP_919214	92	5.00E-020
Cluster 34[a]	2	734	Multidrug Resistance Protein ABC superfamily	BAB85651	399	e-110	XP_463416	365	e-101
SPH C232	1	547	Na^+H^+ Antiporter	XP_476311	33	6.6	XP_476311	33	0.13
SPH_C284	1	341	Perm ease-like ABC transporter	XP_479148	178	6.00E-044	XP_479148	178	8.00E-046
Cluster 35[a]	2	725	Peroxisomal Membrane Anchor Protein PEX14VE	XP_483739	379	e-104	XP_483739	379	e-105
SPH C508	1	568	Phosphatidylinositol/Phosphatidylcholine Transfer Protein	AAU43984	131	2.00E-029	XP_464026	103	7.00E-023
SPH C472	1	741	Predicted membrane Protein	ABB47915	244	2.00E-063	XP_472552	29	2.5

Table 1 (*Continued*)

Sequence ID	Clones contained	Length	Annotation	Top Blastx alignment			Top rice reference alignment		
				Accession	Score	Value	Accession	Score	Value
SPH C430	1	360	Proline Transporter	BAB69951	63	4.00E-009	XP_476343	56	4.00E-009
SPH C136	1	359	Protein Involved in Vacuolar Polyphosphate Accumulation	XP_473572	199	3.00E-050	XP_473572	199	3.00E-052
SPH C49	1	642	Secretory Carrier Membrane Protein	XP_463053	283	2.00E-075	XP_463053	280	5.00E-076
SPH C68	2	565	SNARE Syntaxin	XP_506177	225	6.00E-058	XP_506177	225	1.00E-059
SPH C288	1	330	Sugar Transporter Protein	NP_922890	201	9.00E-051	NP_922890	201	1.00E-052
SPH C399	1	486	Sulfate Transporter	XP_470584	123	3.00E-027	XP_470584	123	6.00E-029
SPH_C251	1	489	Vacuolar Targeting Receptor	AAF80450	329	2.00E-089	XP_479541	301	1.00E-082
Unclassified proteins[b]									
SPH C17	1	721	CEO Protein	NP_922878	311	2.00E-083	NP_922878	311	3.00E-085
SPH_C270	1	422	r40c2 Protein	XP_479572	148	7.00E-035	XP_479572	148	1.00E-036
Viral genes									
Cluster 36[a]	2	715	Integrase core domain	XP_471366	318	9.00E-086	XP_471366	318	2.00E-087
SPH C405	1	450	Mosaic Virus Helicase Domain Binding Protein	AAP80658	198	6.00E-050	NP_910388	150	2.00E-037
SPH C431	1	354	Potyviral Helper Component Protease	BAD45803	165	4.00E-040	XP_467700	121	8.00E-029
Cluster 37[a]	6	703	Retrotransposon Gag-pol Protein	AAN40025	238	1.00E-061	XP_471876	234	3.00E-062
Cluster 38[a]	16	1096	Retrotransposon Protein	BAA22288	478	e-133	NP_909603	463	e-131
Cluster 39[a]	4	733	Retrotransposon Protein TNP2-like	AAM94290	291	2.00E-077	NP_919490	289	1.00E-078
Cluster 40[a]	10	727	Retrotransposon Protein Ty3-gypsy-like	ABA99509	235	1.00E-060	XP_468628	234	5.00E-062
SPH C275	2	378	Retrotransposon Protein Bilby-like	AAU90169	126	2.00E-028	NP_920308	100	2.00E-022
Cluster 41[a]	5	725	Retrotransposon Protein CACTA-Family	ABA96332	99	1.00E-019	XP_472103	96	2.00E-020
SPH_C52	1	633	Retrotransposon Protein Rim2	XP_482244	37	0.66	XP_482244	37	0.01
Cluster 42[a]	4	609	Retrotransposon Protein Ty1-copia	BAA22288	269	6.00E-071	XP_471538	263	6.00E-071
Cluster 43[a]	6	741	RVT Reverse Transcriptase	XP_475035	328	9.00E-089	XP_475035	328	1.00E-090
SPH_C101	1	479	Transposase Protein Gypsy-type	XP_474491	104	9.00E-022	XP_474491	104	2.00E-023
Cluster 44[a]	5	737	Transposase Protein TNP2-like	AAM94290	327	2.00E-088	NP_920023	322	1.00E-088

Genes in bold face type have had their expression pattern tested via Northern blot.

[a] Please see supplemental data for cluster details.

[b] For the sake of brevity 60 unidentified genes with homologous EST's and 226 unknown genes with no hits on homologous EST's have been omitted, see online supplemental information.

DNA sequencer (ABI Prism, USA) using the T7 promoter primer.

2.6. Analysis of differentially expressed genes

The raw sequence information was cleaned and trimmed of contaminating vector sequences using the Lucy software package [20]. Sequences of low confidence (abundant N), extremely short (under 100 bp) or comprised of repetitive base composition were discarded. The remaining sequences were aligned and theoretical consensus sequences constructed using the The Institute for Genomic Research (TIGR) Assembler 2 package [21]. The resulting unique sequences were subjected to several BLAST, BLASTX [22] and RPS-BLAST [23] searches against GenBank and the CDD [24] databases to provide annotation information. The results of these searches were used to manually place the unique sequences into different categories based on proposed function.

2.7. Northern blot analysis of the transcripts

Candidate genes were selected based on annotation and their frequencies in the SSH library, and subjected to further expression analysis by Northern blot analysis (highlighted in bold in Table 1). Ten micrograms of total RNA isolated from stems and leaves of *L. temulentum* was electrophoretically separated on 1.2% denaturing formaldehyde agarose gels and blotted onto a Hybond N nylon membrane [25]. Inserts of selected cDNA were PCR-amplified using T7 and SP6 primers and a standard three-step protocol with an annealing temperature of 55 °C. The amplified fragments were precipitated and re-hydrated at a concentration of 25 ng/mL. ^{32}P-labeled DNA probes for Northern analysis were generated from PCR-amplified fragments of selected cDNA using the Ambion DECAprime II DNA labeling kit (Ambion, USA). The membranes were hybridized with ^{32}P-labeled probes in a solution containing 50% formamide, Denhardt's solution, 0.1% (w/v) SDS, 6× SSPE and 100 mg/mL denatured salmon-sperm DNA overnight at 42 °C. Filters were initially washed in 2× SSC and 0.1% SDS at 42–50 °C for 30 min, then subjected to a wash starting at 50 °C and gradually cooled to room temperature with shaking briefly in 0.2× SSC and 0.1% SDS at RT before autoradiography.

3. Results

To evaluate the utility of *L. temulentum* in the study of post-harvest gene expression the aerial portions of mature (hard dough stage) seed bearing plants were subjected to simulated grass seed harvest. The plants were cut at ground level and piled

6–8 in. high in simulated straw heaps. The heaps were place in an open plastic tub to reduce evaporative water loss from the bottom and sides of the piles, simulating conditions found in the large continuous windrows (straw piles) observed in the field after harvest. Over the course of 48 h, samples representing a cross-section of the straw pile were taken, seed heads were removed and the remaining stalks and leaves collected for evaluation. These tissues were used to create a PCR-based subtraction library to investigate the post-harvest induced gene expression in grasses. After subtraction, the resulting gene fragments were cloned and 768 random white colonies were selected and sequenced. After contiguous assembly 598 unique sequences were identified with an average contiguous size of 875 base pairs. Sequences were subjected to blast searches and the tentative annotation of each gene fragment was determined. Blast searches of the translated gene fragments against the protein database and the rice genome revealed high homology to many known genes.

The distribution of genes found in the library is shown in Table 1. The table has been categorized to highlight genes from 15 different functional categories based on our annotation. Blast searches of GenBank and protein motif scans using rpsblast (Reverse Position Iterative Blast) were performed for each gene and this information was used to determine their identity. Table 1 contains an ID number for each unique gene, the number of cloned sequences that were used to generate the contig or cluster and the final length of the gene fragment. Table 1 also includes the corresponding Accession numbers for the top matches derived from searches of GenBank via blastx (six frame translation versus protein database) and blastn (nucleotide versus nucleotide) of the rice genome. For brevity 286 genes with no known annotation have been omitted from Table 1 (see online Supplementary data).

The analysis showed that over 10% of the sequences (65) detected in this library were from genes associated with plant stress responses, detoxification and protein degradation and folding. Another 12% of the observed genes (76) were closely related to general metabolism. Many of the genes may also be associated with indirect stress response such as increasing important processes like glycolysis. Nearly 9% of the sequences (53) appear to be viral genes and are likely to be transposon related. Extensive transposon expression is often associated with plant stress [26,27]. Table 2 shows the final number of unique sequences found in each category.

Nearly half of the observed stress related genes were strongly associated with dehydration. We found 10 clones (comprised of four contigs) for dehydrins; 14 clones (comprised of seven contigs) for Peptide Chain Release Factor and three clones (comprised of three contigs) for sucrose synthase. Such genes are commonly involved in reducing the initial impact of the osmotic stress. Approximately 7% of the sequences encoded genes involved in protein turnover and maintenance. Protein degradation plays an important role in the remobilization process and has been shown to be an essential element of the senescence process [28,29,30].

Many of the sequences appeared to form clusters corresponding to the same gene, as seen for rab25 dehydrin. Our analysis found that clusters with the same gene annotation, although similar, contained different sequences. The most probable explanation is that different sections of the same gene formed multiple contigs, due to gaps caused by missing sequence information. This behavior is likely due to the fact that the SSH libraries are constructed such that the average fragment size is from 500 to 600 bp, and therefore insufficient for whole EST spanning, potentially resulting in incomplete EST assembly. Additionally, the conservative parameters used to assemble the contigs prevented sequences from becoming merged in a single contiguous sequence despite as much as 95% (in some cases) identity to each other. Conversely, poorly matching sequences may represent multiple isoforms or family members of the same gene.

Table 2
Distribution of clones by functional category

Category	Total sequences
Detoxification	10
Energy transfer	6
General metabolism	76
Maintenance genes	28
Photosynthesis sp.	2
Plastid/mitochondrial genome	43
Protein degradation, folding and transport	40
Ribosomal	5
Signal transduction	15
Stress related	15
Transcription factor	18
Transport and membrane bound	29
Unclassified proteins	55
Unidentified proteins	203
Viral genes	53
Unique sequences	598

In addition to genes that have probable roles in stress responses, 76 clones were associated with general metabolism. Genes, such as amino acid tRNA's, glyoxysomal citrate synthase and glutamine-dependent asparagine synthetase, demonstrate the probable need for additional re-enforcement of critical metabolic pathways during stress. In addition to critical processes such as glycolysis, there were a number of genes including 4-coumarate-CoA ligase and fatty-acyl-CoA synthase that had no clear relation to dehydration stress responses. Our analysis found that over 40% (286) of the total sequenced clones corresponded to novel gene fragments with no previously identified function, although in many cases, there were strong matches to other EST and genomic sequences in GenBank. Consequently, the specific annotation of these sequences could not be determined. For the sake of brevity many of these sequences were omitted from Table 1. It is possible that these ''unknown'', yet expressed, candidate genes play an important role in stress responses. The entire collection of sequences were submitted to GenBank (Accession numbers EL738022-EL738619, please refer to online supplemental data for correlation of GenBank accession numbers and Sequence ID in Table 1).

In order to evaluate the validity of the post-harvest library, the expression of 14 gene fragments from different categories

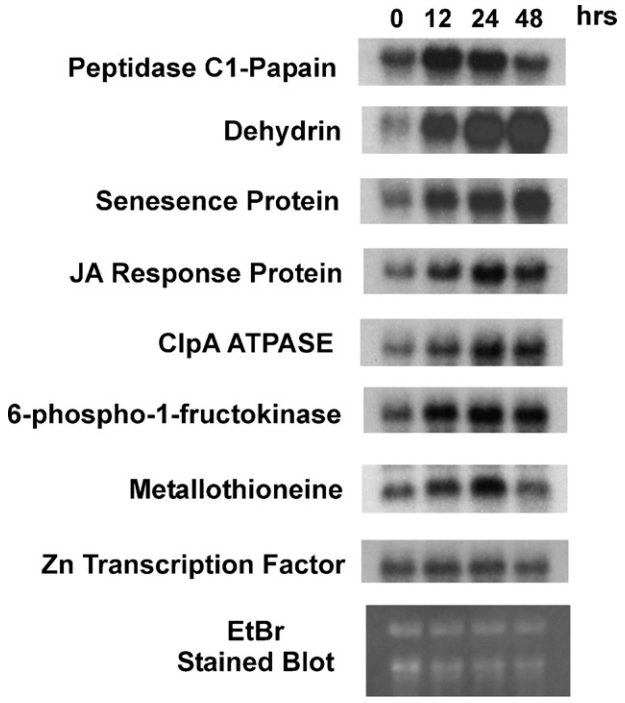

Fig. 1. Northern blot analysis of post-harvest induced expression from selected genes isolated from the subtractive library from *Lolium temulentum* plants. Peptidase C1-papain (Cluster 19, SPH_C514), dehydrin (Cluster 29, SPH_C124), senescence protein (SPH_C224), JA response protein (SPH_C216), ClpA ATPase (Cluster 16, SPH_C333), 6-phospho-1-fructokinase (SPH_C76), metallothioneine (Cluster 2, SPH_C455) genes and Zn transcription factor (SPH_C267) were selected for evaluation. Total RNA was extracted from stems and leaf tissue from plants that had been subjected to harvest stress for the indicated number of hours (0, 12, 24 or 48 h). Each lane of the agarose gel was loaded with 10 μg total RNA. Equal loading of RNA per lane was confirmed by visualization with ethidium bromide of the gel before blotting (data not shown), the nylon membrane after blotting and by the constitutively expressed gene Zn transcription factor (SPH_C267).

of the library (highlighted in bold in Table 1) were analyzed by Northern blot (Fig. 1). It should be noted that the hybridization results of the Northern blots may represent the contribution of one or more gene family members. However, observable differential expression patterns would strongly reinforce the expression predictions of the SSH library data. To this end, several gene fragments were arbitrarily selected based on function, frequency and category. Peptidase C1-papain (Cluster 19, SPH_C514), dehydrin (Cluster 29, SPH_C124), senescence protein (SPH_C224), JA response protein (SPH_C216), ClpA ATPase (Cluster 16, SPH_C333), 6-phospho-1-fructokinase (SPH_C76) and metallothioneine (Cluster 2, SPH_C455) genes were shown to have the most pronounce induction during post-harvest stress (Fig. 1). While clones for the peptide release factor (SPH_C213), metalloprotease (SPH_C396), lipoxygenase (SPH_542) and sucrose synthase (SPH_C172) all slowed slight induction to post-harvest stress (data not shown), and lon protease (SPH_C366) (data not shown), cysteine proteinase inhibitor (SPH_C515) (data not shown) and Zn transcription factor (SPH_C267) showed no induction by Northern blot analysis.

As shown in Fig. 1, all the gene fragments shown, except the Zn transcription factor (loading control), were responsive to the post-harvest stress stimulus. The dehydrin and senescence protein gene fragments were included because dehydration stress and cell senescence are two expected outcomes of post-harvest stress. Additionally, we selected two classes of proteases, a C1 papain type and a ClpA protease subunit, because they have a putative role in protein recycling under the forced senescence found in post-harvest tissue. The 6-phospho-1-fructokinase and metallothioneine gene fragments have less well-defined expression patterns in relation to plant post-harvest stress. Each gene fragment that was analyzed by Northern blot had low levels of expression prior to harvesting. This is not surprising since the plants were displaying signs of the onset of senescence prior to cutting. Both dehydrin and senescence protein gene fragments showed increasing levels of expression throughout the course of the experiment, with dehydrin displaying the most dramatic increase in transcript levels over the 48 h period. The two proteases, JA response protein and the metallothioneine appeared to reach peak expression level after 24 h. The ClpA protease subunit transcript appeared to remain at a constant level for the remainder of the experiment; whereas the expression of C1 papain, JA response protein and metallothioneine decreased after 48 h. The 6-phospho-1-fructokinase message also continued to accumulate throughout the course of the experiment (up to 48 h). The continued expression of this critical metabolic gene, coupled with the somewhat muted expression of C1 papain and metallothioneine at the 48 h mark, may indicate the onset of generalized senescence due to severe stress-based restriction of normal metabolic processes.

4. Discussion

The primary focus of this research was the identification of genes that are up-regulated in *L. temulentum* straw after a simulated grass seed harvest. The results obtained using the SSH approach demonstrated the benefits of this model grass, yielding a high-resolution profile of genes induced by post-harvest stress. The cDNA subtraction strategy identified nearly 600 unique gene candidates for further study. Blast searches of these genes showed that many of the isolated genes had significant homology to known sequences in GenBank. Many of these genes have probable roles in senescence, dehydration stress, and the maintenance of critical cellular metabolic processes. As such, they are likely involved in plant responses to post-harvest stress.

Northern blot analysis of selected gene fragments showed clear post-harvest-regulated-expression of a wide variety of gene types. It was encouraging to find that even genes not typically associated with drought, senescence or wounding such 6-phospho-1-fructokinase and metallothioneine showed highly regulated expression patterns (Fig. 1). It is possible that some of the genes identified in Table 1, which are associated with signal transduction, or are putative transcription factors play an important regulatory role in senescence or plant stress responses in grasses. Of further interest will be the investigation

of the expression patterns and roles played by some of the novel or unknown genes identified in this study.

We felt that it was critical to carefully select a useful model plant to facilitate a detailed profiling of the genes involved in the post-harvest stress response. The sheer number of potential candidates for post-harvest specific genes expression identified in *L. temulentum* is encouraging. Due to the limited research tools available in the study of forage and turf grasses, the development of model systems is of paramount importance. *L. temulentum* with its short generation time, diploid genome and self-fertile nature appears to be an ideal candidate for future application of large scale studies utilizing modern molecular tools. This grass has been shown to be amenable to tissue culture [19], plant breeding [16,17,31–33] and gene expression profiling as demonstrated by this study. The SSH approach provided valuable insight into the expression patterns of genes associated with post-harvest stress.

Our analysis detected a number of genes commonly associated with related plant stresses such as dehydration and senescence in addition to many novel transcripts. Identification of genes that are up-regulated in response to cutting is one of the first steps towards developing a better understanding of post-harvest stress responses. Future experiments will further characterize the temporal and spatial expression of these candidate genes and isolate their promoters. The ultimate goal will be to develop molecular tools that lead to a high level of gene expression in targeted tissues during post-harvest stress but little to no expression in non-senescent tissues.

The identification of post-harvest up-regulated genes, their promoters and their subsequent characterization will lead to molecular and genetic approaches for improvement of forage and turf grass residues. The information presented in this manuscript provides critical baseline information regarding molecular processes initiated by grass harvesting. This information will be useful in future research directed at modifying the molecular composition of grass cell walls after harvesting to improve both the nutritional value for animal consumption and the efficiency of conversion of grass biomass into biofuels. Using this approach, it should be possible to create an improved forage product expressing genes capable of enhancing digestibility after the grass is cut with no discernible loss in stand vigor under normal field growing or seed-producing conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2007.04.001

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